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Acupuncture Research

Electroacupuncture Attenuates Immune-Inflammatory Response in Hippocampus of Rats with Vascular Dementia by Inhibiting TLR4/MyD88 Signaling Pathway*

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ABSTRACT **Objective**: To investigate whether electroacupuncture (EA) alleviates cognitive impairment by suppressing the toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) signaling pathway, which triggers immune-inflammatory responses in the hippocampus of rats with vascular dementia (VaD). **Methods**: The experiments were conducted in 3 parts and in total the Sprague-Dawley rats were randomly divided into 8 groups by a random number table, including sham, four-vessel occlusion (4-VO), 4-VO+EA, 4-VO+non-EA, sham+EA, 4-VO+lipopolysaccharide (LPS), 4-VO+LPS+EA, and 4-VO+TAK-242 groups. The VaD model was established by the 4-VO method. Seven days later, rats were treated with EA at 5 acupoints of Baihui (DV 20), Danzhong (RN 17), Geshu (BL 17), Qihai (RN 6) and Sanyinjiao (SP 6), once per day for 3 consecutive weeks. Lymphocyte subsets, lymphocyte transformation rates, and inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were measured to assess immune function and inflammation in VaD rats. Transmission electron microscopy was used to observe the ultrastructure of nerve cells in the hippocampus. The levels of TLR4, MyD88, IL-6, and TNF-α were detected after EA treatment. TLR4/MyD88 signaling and cognitive function were also assessed after intracerebroventricular injection of TLR4 antagonist TAK-242 or TLR4 agonist LPS with or without EA. **Results**: Compared with the 4-VO group, EA notably improved immune function of rats in the 4-VO+EA group, inhibited the protein and mRNA expressions of TLR4 and MyD88 in the hippocampus of rats, reduced the expressions of serum IL-6 and TNF-α (all $P<0.05$ or $P<0.01$), and led to neuronal repair in the hippocampus. There were no significant differences between the 4-VO+LPS+EA and 4-VO+EA groups, nor between the 4-VO+TAK-242 and 4-VO+EA groups ($P>0.05$). **Conclusions**: EA attenuated cognitive impairment associated with immune inflammation by inhibition of the TLR4/MyD88 signaling pathway. Thus, EA may be a promising alternative therapy for the treatment of VaD.

KEYWORDS electroacupuncture, vascular dementia, immune-inflammation, toll-like receptor 4/myeloid differentiation factor 88 signaling pathway

Vascular dementia (VaD) is a syndrome of cognitive dysfunction caused by cerebrovascular factors, and has become the second common dementia among the elderly in Europe, United States and East Asia.[1] At present, there are no effective treatments for VaD, so exploration of the pathogenesis and treatment methods of VaD requires urgent attention. To improve the quality of life of patients with VaD, an increasing number of complementary and alternative medical treatments have been embraced due to their low toxicity.[2,3] In this vein, electroacupuncture (EA) has been increasingly used as a treatment for VaD.[4]

Our previous research reported that EA improved cognitive function in the four-vessel occlusion (4-VO) rat model of VaD by inhibiting inflammatory cascade.[5] However, further experimental research is required to explore the mechanisms behind the effectiveness of EA treatment on VaD.

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Inflammation plays an important role in the occurrence and development of cerebral ischemia-reperfusion injury (CIRI).[6] Within a few hours after CIRI, the expression of inflammatory factors in ischemic brain cells increases significantly,[7] causing tissue damage through various mechanisms, including microvascular occlusion, vasomotor changes, oxygen free radical production, cytotoxic enzyme release, and chemokine release. Chemokines further activate other cytokines and immune responses to worsen brain damage.[8]

The toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) signaling pathway can mediate inflammation and aggravate ischemic brain injury,[9] but its relationship with VaD is unclear. TLR4/MyD88 plays a vital role in ischemic injury, but few studies have shown that TLR4/MyD88 signals affect the inflammatory response within a potential treatment window after VaD onset. The current study was performed to investigate the effect of EA treatment on the immune function and inflammatory response of VaD rats. Moreover, the relationship between the TLR4/MyD88 signaling pathway and the cognitive function of VaD rats after EA intervention was also explored.

METHODS

Animals

Male Sprague-Dawley rats (180–200 g, specific pathogen-free grade) were provided by Changsha Tianqin Biotechnology Co., Ltd., China (Certificate No. SCXK 2019-0014). All rats were housed at constant room temperature (22±2 ℃) and humidity (60%±5%) under a controlled 12-h light-dark cycle with standard rodent food and water. The experiments were approved by the Ethics Committee for Animal Experimentation of Guizhou University of Traditional Chinese Medicine. All animals were raised in their new surroundings for 7 days before the experiments, and trauma was minimized as much as possible during the experiments.

Establishment of VaD Rat Model

The VaD rat model was established by the 4-VO method.[10] Rats were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium and were placed in the prone supine position. The first cervical vertebral arteries were exposed bilaterally and were cauterized by electrocauterization to form a permanent occlusion. Then the rat was placed in supine position. The common carotid artery was bluntly dissociated through a cervical midline incision and occluded using a non-invasive microvascular artery clamp to block blood flow for 10 min, followed by reperfusion for 1 h. The ischemia-reperfusion process was repeated 3 times. In the sham-operated animals, none of the arteries was electrocauterized or clamped. The successful application of 4-VO was confirmed when (1) the rat's pupils dilated and the irises whitened within 2 min after the 4 vessels were occluded, and (2) the rat's performance on the Morris water maze (MWM) test was impaired.[11] Rats that met the above two criteria were selected as 4-VO rats for subsequent experiments. According to the following formula,[12] the rats were identified whether they had cognitive dysfunction.

$$[(B-A)/A]\%>20\%$$

A-value: the time latency of rats in the sham group; B-value: the time latency of rats in the 4-VO group.

Experimental Design

Experiment Ⅰ

To determine the immune function and anti-inflammation effect of EA treatment, rats were assigned to 5 groups by a random number table: sham, 4-VO, 4-VO+EA, 4-VO+non-EA, and sham+EA groups (n=6 per group). A tetrazolium-based colorimetric assay (MTT assay) and flow cytometry were performed to evaluate the immune function of VaD rats before and after EA treatment. The expressions of inflammation cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) in serum and hippocampus were detected by enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PCR), respectively (Appendix 1A).

Experiment Ⅱ

To observe the potential mechanism of hippocampal inflammation and TLR4/MyD88 signaling in VaD rats before and after EA treatment, rats were randomly assigned to 4 groups: sham, 4-VO, 4-VO+EA, and sham+EA groups (n=6 per group). The expression levels of mRNA and proteins of the TLR4/MyD88 signal pathway and downstream inflammatory cytokines were detected by real-time PCR, Western blot, and ELISA after 7, 14 and 21 days of EA treatment, respectively.

The ultrastructure changes of rat hippocampal neurons were observed by transmission electron microscope (TEM) after 21 days of EA treatment (Appendix 1A).

Experiment Ⅲ

To assess the role of TLR4 in the mechanism of beneficial effects of EA, intracerebroventricular injection of TLR4 antagonist TAK-242 and agonist lipopolysaccharide (LPS) was performed. Rats were randomly assigned to 6 groups, including sham, 4-VO+LPS, 4-VO+LPS+EA, 4-VO+TAK-242, 4-VO, and 4-VO+EA groups (n=6 per group). Cognitive function and proinflammatory cytokine levels were evaluated by the MWM test, Western blot and ELISA, respectively (Appendix 1A).

EA Treatment

Seven days after surgery, rats in the sham+EA and 4-VO+EA groups were treated with EA after anesthesia as follows. Five acupoints of Baihui (DV 20), Danzhong (RN 17), Geshu (BL 17), Qihai (RN 6) and Sanyinjiao (SP 6) were pierced by a 0.3 mm diameter needle (Shanghai Pudong Jinhuan Medical Products Co., Ltd., China). By using an electroinc acupuncture treatment instrument (sdz-Ⅱ, Suzhou Medical Supplies Factory, China), a mild electrical current was applied across the needles with a sparse-dense wave of 2/15 Hz at 1.0 mA for 30 min per day.[13,14] Rat body temperature was maintained at 37±0.5 ℃ with a heating pad during EA treatment. The specific locations and acupuncture depths of the acupoints were determined using the rat acupoint atlas.[15] Detailed acupoint locations and manipulations are shown in Appendixes 1B and 2. Rats in the 4-VO+non-EA group had the same operation as above, except that the acupuncture positions used were located around the 5 acupoints, not located on the meridian route, and did not belong to the traditional 14 meridians. EA and non-EA treatments were performed once per day for a total of 3 weeks. All rats in the other groups were given the same time and same level catching-grasping stimulus without EA treatment.

Intracerebroventricular Injection of LPS and TAK-242

The TLR4 agonist LPS (Cat No. HYD1056, MCE, USA) was dissolved in sterile saline and was administered at 1 μg/10 μL into the lateral ventricle. The TLR4 antagonist TAK-242 (Cat No. HY11109, MCE) was dissolved in 10% dimethyl sulfoxide (DMSO, Cat No. F8110, Sigma-Aldrich, USA) and 40% polyethylene glycol 300 (Cat No. S6704, Selleckchem, USA) and was infused via intracerebroventricular injection at a dose of 0.6 mg/10 μL. Drugs were infused by a microsyringe at a rate of 1 μg/min, and the needle was left for an additional 10 min before withdrawal to prevent reflux. Drug administration was all conducted 30 min before EA treatment.

The operation of intracerebroventricular injection was as follows. Rats were anesthetized with pentobarbital sodium and fixed onto the stereotaxic apparatus (ASI Instruments, USA). A stainless-steel cannula was implanted unilaterally into the lateral ventricle (from the bregma: anterior-posterior –0.8 mm, lateral 1.3 mm, and ventral –3.5 mm). Finally, dental cement was used to fix the cannula onto the skull. Following surgery, animals recovered for 7 days.

MTT Assay

After euthanasia with 10% sodium pentobarbital (0.3 mL/100 g), the rats' spleens were removed and made into single cell suspensions. A separation solution was added in an equal amount, and the second layer of lymphocytes was removed with a pipette. The cell suspension was then suspended in RPMI 1640 medium (WH01112007XP, Procell, China) and centrifuged at 400×g for 10 min. Cells were added to a cell culture plate in RPMI 1640 medium with concanavalin A (final concentration 5 mg/L). Plates were incubated in 5% CO_2 at 37 ℃ for 72 h, followed by the addition of 0.5 mg/mL MTT solution to each well and incubation for 4 h. The supernatant was discarded and DMSO (SHBL0852) was added to each well, followed by slight vibration for 10 min. The optical density (OD) at 570 nm was measured using a microplate reader (Spectra max PLUS 384, Molecular Devices, USA). The stimulation index (SI) was used to represent the lymphocyte transformation rate in the spleen. SI = average OD of phytohemagglutinin stimulus wells/average OD of control wells.

Flow Cytometry

Heparin-treated noncoagulated blood was collected from rats in each group through the abdominal aorta. After incubation in red blood cell lysis buffer (S7418, Tiangem, China), lymphocytes were collected and stained using the following labeled monoclonal antibodies: APC-labeled anti-rat CD3 (Cat No. 201414, Biolegend, USA), FITC-labeled mouse

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anti-rat CD4 (Cat No. 561834, BD Biosciences, USA), and PE-labeled mouse anti-rat CD8 (Cat No. 554857, BD Biosciences). The stained cells were used in calculating the relative percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD4^+/CD8^+$ T-lymphocytes by a flow cytometry (Cytoflex, Breckman, USA).

Real-Time PCR

Total RNA was extracted from the hippocampus using TRIzol reagent (VS18061730, Hefei Bomei Biotechnology Co., Ltd., China) in accordance with the manufacturer's instructions. The mRNA samples were reverse transcribed to cDNA. The primer sequences of TLR4, MyD88, IL-6, TNF-α, and β-actin amplification are shown in Appendix 3. The PCR cycle was as follows: 95 ℃ for 30 s, 45 cycles of 95 ℃ for 5 s, 55 ℃ for 30 s, and 72 ℃ for 30 min, PikoReal 96 software (Thermo Fisher Scientific, USA) was used to analyze the threshold cycle (Ct) value of each test sample in the PCR process. The relative mRNA expression levels of each target were calculated using $2^{-\triangle\triangle Ct}$.

ELISA

Blood was taken from the abdominal aorta of the rats, and the serum homogenate was used to detect the concentration of IL-6 (ZC-36404) and TNF-α (ZC-37624) using commercially available ELISA kits (both from Shanghai Zhuo Cai Biological Technology Co., Ltd., China) according to the manufacturer's instructions.

TEM

After sacrifice, rat hippocampus was dissected, pre-fixed with 3% glutaraldehyde, and then fixed with 1% osmium tetroxide (GP18456, Leica, USA). Acetone was used to dehydrate the tissues. Then the sample were infiltrated for 40 min, embedded and solidified, and the samples were cut into 50-nm-thick ultrathin sections using an ultramicrotome (EM UC7, Leica). The sections were dyed with uranyl acetate (GS02624) and lead citrate (GZ02616), both from Beijing Zhongjing Scientific Instrument Technology Co., Ltd., China, for 20 min at room temperature, and the samples were observed with TEM (JEM-1400PLUS, Japan).

Western Blot

Hippocampal cells from each group were lysed with radioimmunoprecipitation assay lysis buffer (P0013, Beyotime, China), and the protein content was determined by the quinolinic acid method. Each sample (40 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PG112, Shanghai Ya Enzyme Biological Technology Co., Ltd., China), and the proteins were transferred to polyvinylidene fluoride membranes (ISEQ00010, Sigma-Aldrich). After 50 g/L nonfat dried milk was blocked for 2 h, primary antibody TLR4 (Cat No. AF7071, Affinity, USA), MyD88 (Cat No. AF5195, Affinity, USA), β-actin (Cat No. AC026, Abclonal Biotechnology Co., Ltd., China) rabbit anti-mouse polyclonal antibody were added, incubated overnight at 4 ℃. The membranes were washed and incubated with horseradish peroxidase-labeled secondary antibody [AB6721, British Abcam-Abcam (Shanghai) Trading Co., Ltd. China], washed again, and developed using enhanced chemiluminescence reagent (KF001, Affinity, USA). Each membrane strip was exposed and scanned with a chemiluminescence gel imager 5200 (Shanghai Tianneng Technology Co., Ltd., China).

MWM Test

To assess spatial learning and memory, the MWM test was used as described previously after treatment with agonists or inhibitor. The circular water-maze pool was 160 cm in diameter and was divided into 4 quadrants. For acquisition training, the rats were trained for 6 consecutive days. The rats were allowed 90 s to find the hidden platform. If they failed to locate the submerged platform, the rats were guided to the platform and allowed to stay there for 10 s. On the 7th day, a probe trial was conducted by removing the platform from the pool. The rats were left to swim freely for 90 s. Time latency of 1–6 days and platform crossings on the 7th day in the target quadrant were recorded.

Statistical Analysis

SPSS Statistics for Macintosh, Version 25.0 (IBM Co. Ltd., USA) was used for data analysis. Data were represented by mean ± standard deviation ($\bar{x} \pm s$). Data that satisfied a normal distribution were compared by one-way analysis of variance among multi-groups. Least significant difference test was used for uniform variance, and Tamhane's T2 test was used for uneven variance. While Kruskal-Wallis test was used for data satisfying non-normal distribution. The significant level was $P<0.05$.

RESULTS

EA Improved Immune Function in 4-VO Rats

As shown in Figure 1, the $CD3^+$, $CD3^+CD4^+$, and $CD4^+/CD8^+$ levels in the 4-VO group were lower than

those in the sham group (P<0.01), whereas $CD3^+CD8^+$ level was higher than that in the sham group (P<0.01). EA treatment significantly ameliorated the insufficient expression of $CD3^+$, $CD3^+CD4^+$ and $CD4^+$/$CD8^+$ (P<0.05), but reversed the over-expression of $CD3^+CD8^+$ induced by the 4-VO operation (P<0.05). The lymphocyte transformation rate was significantly reduced in the 4-VO group (P<0.01), but the reduction was inhibited by EA (P<0.05). However, no significant differences were observed between the 4-VO and 4-VO+non-EA groups (P>0.05) and between the sham and sham+EA groups (P>0.05).

EA Alleviated Inflammatory Cytokines in 4-VO Rats

Compared with the sham group, the mRNA and protein levels of IL-6 and TNF-α were all elevated in the 4-VO group (P<0.01), but those were significantly decreased in the 4-VO+EA group (P<0.01). There was no significant difference between the 4-VO and 4-VO+non-EA groups (P>0.05), or between the sham and sham+EA groups (P>0.05, Figure 2).

EA Repaired Hippocampal Neuron Damage in 4-VO Rats

The hippocampus nerve cells from rats in the sham and sham+EA groups had normal morphology and intact structures. The nuclei were oval, the nuclear membranes were intact, the chromatin in the nuclei was evenly distributed, the nuclear membranes were intact without dissolution, and the cytoplasms were rich in organelles, such as mitochondria, rough endoplasmic reticulum, and ribosomes. The hippocampal nerve cells from rats in the 4-VO and 4-VO+non-EA group rats had abnormal nuclear morphology, with increased heterochromatin, reduced numbers of organelles, and disordered arrangement. Mitochondrial cristae appeared broken and missing, and the endoplasms were expanded. The cell membranes were partially ruptured and the cell contents were lost. The hippocampal neuron structure of the 4-VO+EA group rats was relatively complete, as nuclear membranes were invaginated, and the cavitation and expansion of mitochondria and endoplasmic reticulum were significantly lighter than those in the 4-VO group (Figure 3).

EA Suppressed Activation of TLR4/MyD88 Signaling Pathway

As shown in Figure 4, compared with the sham group, the protein and mRNA levels of TLR4 and MyD88 were significantly elevated in the 4-VO group at 7, 14 and 21 days (P<0.05 or P<0.01), while this increase was inhibited by EA in the 4-VO+EA group at 14 and 21 days (P<0.05 or P<0.01). The levels of IL-6 and TNF-α in the hippocampus and serum were increased in the 4-VO group compared with the sham

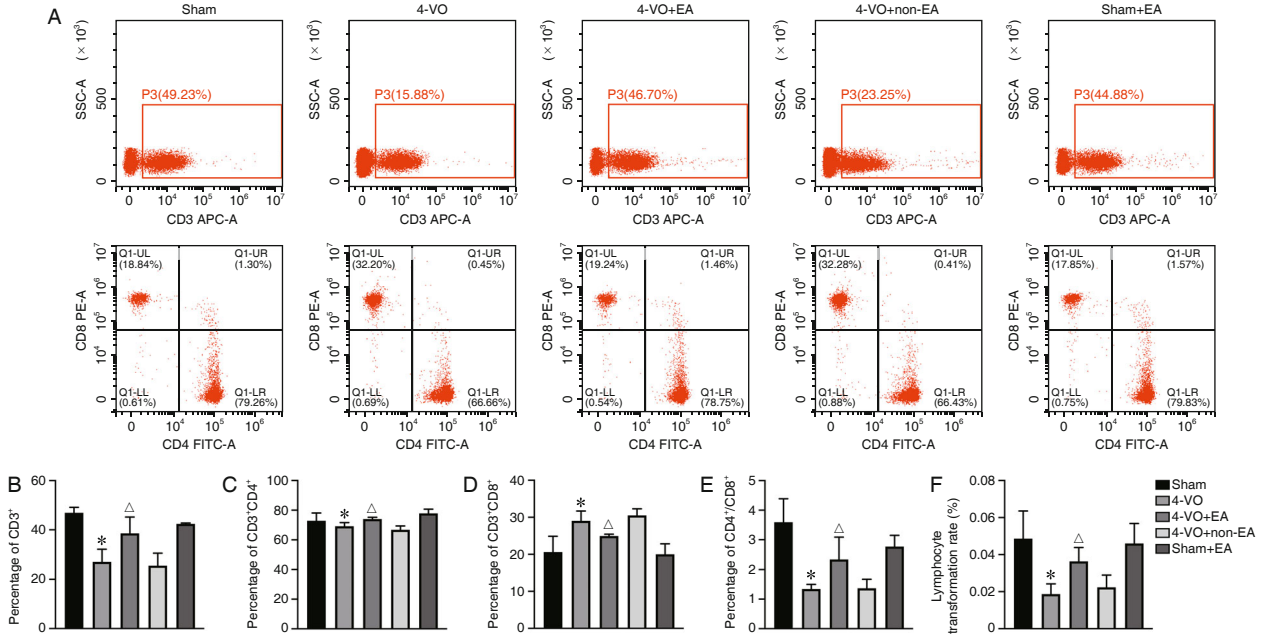

Figure 1. EA Improved Immune Function of 4-VO Rats ($\bar{x} \pm s$)

Notes: A: representative scatters plot shows the percentage of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells in blood of 4-VO rats; B–E: bar graph shows the percentage of T lymphocyte subsets in blood of 4-VO rats; F: lymphocyte transformation rate in spleen of 4-VO rats. *P<0.01 *vs*. sham group; △P<0.05 *vs*. 4-VO group

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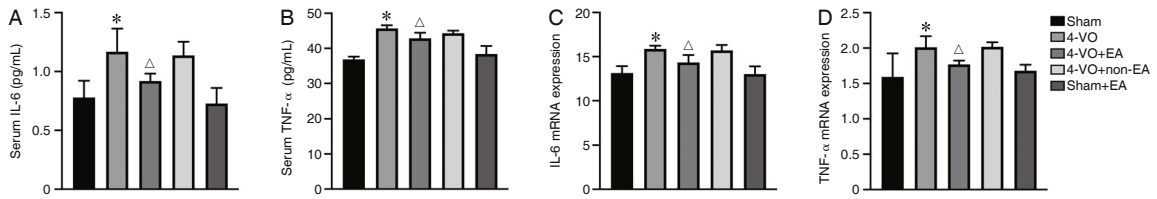

Figure 2. EA Reduced Inflammatory Response in 4-VO Rats ($\bar{x} \pm s$)

Notes: *P<0.01 *vs*. sham group; △P<0.01 *vs*. 4-VO group

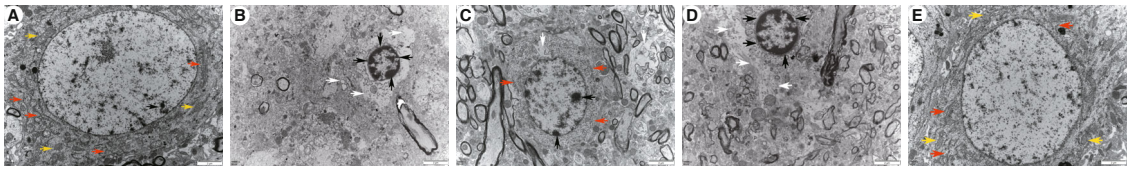

Figure 3. Transmission Electron Microscope Image of Hippocampal Slice (×10,000)

Notes: A: sham group, B: 4-VO group, C: 4-VO+EA group, D: 4-VO+non-EA group, E: sham+EA group. The black arrows indicate mitochondria, the red arrows indicate rough endoplasmic reticulum, the yellow arrows indicate heterochromatin, and the white arrows indicate the lost contents.

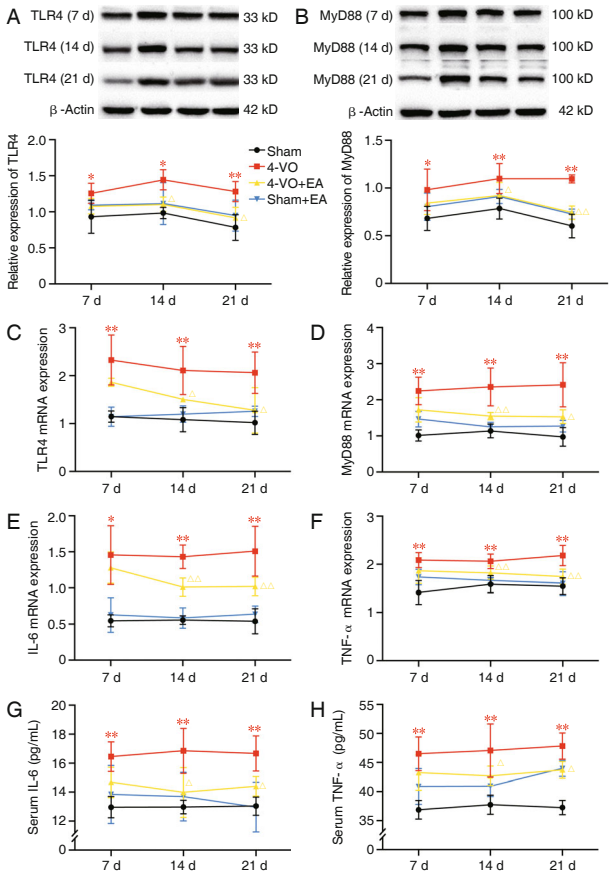

Figure 4. EA Suppressed Expressions of TLR4, MyD88, IL-6, and TNF-α in 4-VO Rats ($\bar{x} \pm s$)

Notes: A and B: representative Western blot bands and protein expressions of hippocampal TLR4 and MyD88 in each group after EA treatment; C–F: mRNA expressions of TLR4, MyD88, IL-6, and TNF-α in hippocampus of each group after EA treatment; G and H: expression levels of IL-6 and TNF-α in serum of each group after EA treatment. *P<0.05, **P<0.01 *vs*. sham group; △P<0.05, △△P<0.01 *vs*. 4-VO group

group (P<0.05 or P<0.01), while these levels in the 4-VO+EA group were significantly decreased compared with the 4-VO group at 14 and 21 days (P<0.05 or P<0.01). However, no significant difference was found between the sham and sham+EA groups (P>0.05).

TLR4 Antagonist Mimicked Neuroprotective Effects of EA, and TLR4 Agonist Reversed Beneficial Effects of EA

Compared with the sham group, the expressions of TLR4 and MyD88 protein in the hippocampus and the levels of IL-6 and TNF-α in the serum were significantly increased in the 4-VO and 4-VO+LPS groups (P<0.05 or P<0.01). Compared with the 4-VO group, the protein expressions of TLR4 and MyD88 and the levels of IL-6 and TNF-α were all decreased in the 4-VO+LPS+EA, 4-VO+TAK-242 and 4-VO+EA groups (P<0.05 or P<0.01). No significant difference was found between the 4-VO+LPS+EA and 4-VO+EA groups, the 4-VO+TAK-242 and 4-VO+EA groups, the 4-VO+LPS+EA and 4-VO+TAK-242 groups (P>0.05, Figure 5).

EA Improved Cognitive Function of VaD Rats through TLR4/MyD88 Signaling Pathway

As shown in Figure 6 and Appendix 4, compared with the sham group, the escape latency of rats in the 4-VO and 4-VO+LPS groups was prolonged (P<0.05 or P<0.01), and the number of crossings through the original platform position was reduced on the 7th day (P<0.01). Compared with the 4-VO group, the escape latency of rats in the 4-VO+TAK-242 and 4-VO+EA groups was significantly shorter (P<0.05), and the number of platform crossings increased (P<0.05). However, there was no significant difference between the 4-VO+LPS+EA and 4-VO+EA groups, the 4-VO+TAK-242 and 4-VO+EA groups, the

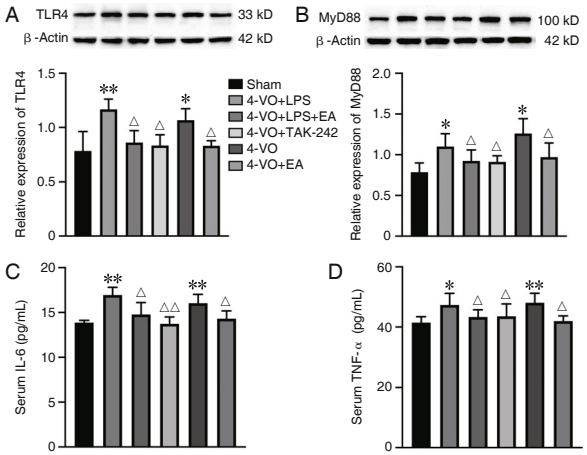

Figure 5. TAK-242 Mimicked Effects of EA on Inflammation Inhibition, and LPS Reversed These Effects of EA ($\bar{x} \pm s$)

Notes: A and B: protein levels of TLR4 and MyD88 in hippocampus. C and D: contents of IL-6 and TNF-α in serum. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. sham group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. 4-VO group

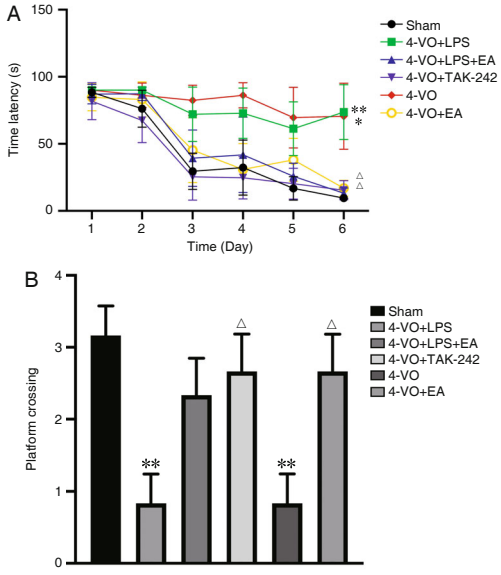

Figure 6. Time Latency and Platform Crossings in MWM Test ($\bar{x} \pm s$)

Notes: $^{*}P<0.05$, $^{**}P<0.01$ *vs*. sham group; $^{\triangle}P<0.05$ *vs*. 4-VO group

4-VO+LPS+EA and 4-VO+TAK-242 groups ($P>0.05$).

DISCUSSION

In this study, experiments were conducted in 3 parts to explore the TLR4/MyD88 signaling pathway and immune-inflammatory responses after cerebral ischemia. Our results showed that EA treatment significantly enhanced immune function and reduced the levels of over-expressed inflammatory factors in the hippocampus and serum of VaD rats. EA inhibited the TLR4/MyD88 signaling pathway and inflammatory response and reduced the number of injured nerve cells in the hippocampus. Thus, the cognitive dysfunction in VaD rats was improved. The TLR4 agonist LPS was used to activate TLR4 in the hippocampus of VaD rats. EA and TAK-242 had similar inhibitory effects on the TLR4/MyD88 signaling pathway and downstream inflammatory factors.

VaD is a progressive disease that affects cognitive abilities and is caused by reduced blood flow to the brain.[16] EA is an effective stimulator of the central nervous system (CNS), and plays an important role in the recovery of learning and memory functions after brain injury. One part of the EA signal passes through the afferent nerve to the posterior horn of corresponding segment of spinal cord and then to the internal organs, which play a regulatory role.[17] The other part of the EA signal passes through the spinal cord. The horns join to the cerebral cortex, strengthening the interference, inhibition and replacement of the excitement of pathological stimulus by CNS, then adjust the function state of the organs through regulation of nerve-humoral fluids, promotion of the body's metabolism, and improve the immune defense ability.[18]

There are many reports on EA treatment for ischemic cerebrovascular disease, and good efficacy has been achieved in the treatment of VaD.[19,20] In this experiment, we selected 5 acupoints commonly used in clinical practice. The results suggested that EA enhanced the transformation rate of splenic lymphocytes in VaD rats, regulated the balance of T lymphocyte subsets, and maintained homeostasis of the cellular immune system. IL-6 mainly activates T cells and participates in the release of other immune cells. It plays an irreplaceable role in immune regulation, inflammatory response and hematopoietic regulation.[21] Immune function can be measured by detecting changes in serum IL-6 levels.[22] TNF-α mediates a cellular immune response, mainly by stimulating the proliferation and activation of T lymphocytes and enhancing the cytotoxicity of T lymphocytes. It plays an immune role and participates in the process of inflammatory disease.[23] The TEM results showed that there was severe neuronal degeneration and increased cellular debris in the hippocampus of 4-VO rats. However, after a longer EA treatment time, the neuronal damage in the hippocampus was significantly improved and the neurons appeared healthier. This experiment suggested that EA reduced the expressions of IL-6

and TNF-α, played a role in protecting neurons, and ultimately reversed the effects of excessive inflammation in the hippocampus and serum of VaD rats.

TLR4 is one of the TLRs family members, which are pattern-recognition receptors and are mainly expressed in a variety of immune cells.[24] After activation, they can stimulate release of cytokines and chemokines to regulate innate immunity and acquire immune response.[25] Research on the mechanism of TLR4 action in cerebral ischemia has increased in the past two years. At present, the relationship between TLR4 and cerebral ischemia is relatively clear,[26] as it is activated in response to endogenous danger signals released after ischemic brain injury.[27] The extracellular segment of TLR4 consists of 24 leucine-rich repeats, which is the ligand-bingding site. There is a conserved sequence in the intracellular segment that has homology to the conserved sequence of the human IL-1 receptor intracellular segment, also known as the toll/ IL-1 receptor domain.[28,29] After TLR4 recognizes its specific ligand, it binds to a toll/ IL-1 receptor domain–containing adapter protein, such as MyD88. Activated MyD88 leads to an increase in the expression of inflammatory factors IL-6 and TNF-α through different activation pathways, which promotes an inflammatory response and leads to tissue damage.[30,31] TLR4 participates in ischemic brain injury by mediating the inflammatory response, and it directly or indirectly causes nerve cell death when activated.[32] Blocking TLR4 restores the proliferation of nerve cells, and reduces inflammation after ischemic brain injury.[33]

Our results showed that the expressions of TLR4 and MyD88 and activation of the downstream inflammatory factors IL-6 and TNF-α in the hippocampus and serum of rats did not decrease significantly on the 7th day after EA treatment, but there was a significant decrease on the 14th and 21st days of EA treatment. Therefore, the therapeutic effect of EA required at least 2 treatment cycles. The ultrastructural observations of rat hippocampus suggested that EA reduced neuronal edema and improved the structure of damaged neurons. Therefore, the TLR4/MyD88 signaling pathway may serve as a potential target of EA to regulate immune inflammation in VaD.

To verify that the TLR4/MyD88 signaling pathway plays a major role in the effectiveness of EA treatment in VaD rats, the specific TLR4 inhibitor TAK-242 was selected to block TLR4 signaling, thus mediating the expression of inflammatory cytokines, and protecting the brain from ischemia.[34] We found that TAK-242 had similar effects to EA. The TLR4 receptor agonist LPS is the most typical pathogen-related activator of TLR4 and is a powerful inducer of inflammation. LPS activates the innate immune system through TLR4, leading to production of many proinflammatory factors.[35] In the healthy rats, we used intracerebroventricular injection of LPS before each EA treatment in place of the 4-VO model. The TLR4/MyD88 signaling pathway and its downstream factors, IL-6 and TNF-α, were activated in LPS-treated rats. After EA treatment, the expression of the TLR4/MyD88 pathway was significantly reduced, and the cognitive function of rats was improved.

In conclusion, we observed that EA treatment improved immune function and inflammatory response in VaD rats, which may be related to the negative regulation of TLR4/MyD88 signaling pathway in the hippocampus. This study provides a new perspective for understanding the mechanism of EA action and implies that EA has potential as a complementary therapy for VaD.

Conflict of Interest

All authors reported no conflicts of interest and no financial relationships with any companies that may have an interest in the information contained in the manuscript.

Author Contributions

Bu Y and Li WS conducted experimental research, collected the test data, elucidated the results. Bu Y drafted the manuscript. Lin J, Sun QY, Wei YW and Zhu SJ assisted animal tests of this study. Tang ZS planned the research and interpreted the results. All authors read and approved the final manuscript for submission.

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